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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 49/02, C07D 257/02
A61K 43/00

A2

(11) International Publication Number: WO 93/20852

(43) International Publication Date: 28 October 1993 (28.10.93)

(21) International Application Number: PCT/US93/03483

(22) International Filing Date: 13 April 1993 (13.04.93)

(30) Priority data:
07/868,078 13 April 1992 (13.04.92) US

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(81) Designated States: AU, CA, JP, NO, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: PROCESS FOR PREPARING MACROCYCLIC CHELATING AGENTS AND FORMATION OF CHELATES AND CONJUGATES THEREOF

(57) Abstract

The present invention is directed to an improved process for preparing macrocyclic chelating agents and for conjugating the macrocyclic chelating agents to biological molecules.

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PROCESS FOR PREPARING MACROCYCLIC CHELATING AGENTS AND FORMATION OF CHELATES AND CONJUGATES THEREOF

The present invention relates to a process for preparing isothiocyanato functionalized macrocyclic chelating agents and to a process for conjugating the macrocyclic chelating agents to biological molecules.

Metal ions may be attached to biological molecules by means of bifunctional chelating agents. Such chelating agents are compounds which contain a metal-binding moiety which forms a chelate with metal ions and a second functional group, which is chemically reactive in nature and is capable of forming a covalent bond with biological molecules. The reactive functionality is usually one of the various known useful chemically reactive groups such as bromoacetyl group, a diazonium ion, an isothiocyanate or a carboxylic acid derivative, the reactive functionalities being capable of binding to an amino acid of a protein (e.g., the lysine moiety of an antibody). The biological molecules usually recognize distinctive external or internal cell markers and, thus, act as target directing groups for the metal ion.

When the bifunctional chelating agent is covalently attached to an antibody having specificity for cancer or tumor cell epitopes or antigens, radionuclide chelates of such antibody/chelating agent conjugates are useful in diagnostic and/or therapeutic applications as a means of conveying the radionuclide to a cancer or tumor cell. See, for example, Meares et al., *Anal. Biochem.*, 142, 68-78 (1984); and Krejcarek et al., *Biochem. and Biophys. Res. Comm.*, 77, 581-585 (1977).

Isothiocyanato functionalized derivatives of ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) are reported in the literature and are being used to conjugate radioactive isotopes to antibodies. For example see Gansow et al., *Inorg. Chem.*, 25, 2772-2781 (1986); Meares et al., *Anal. Biochem.*, 142, 68-78 (1984); and U.S. Patent 4,454,106.

When using short-lived radionuclides, it is desirable to chelate the radionuclide to the target directing group as close as possible to the time of injection into the patient to provide maximum specific radioactivity and minimum degradation of the radioimmunoglobulin. When using chelating agents such as EDTA and DTPA, the rapid sequestration of metal ions by these chelating agents allows the preparation of the radionuclide/antibody/chelating agent conjugates (referred to herein as conjugates) to be prepared by activating the chelating agent, reacting the chelating agent with an antibody and then chelating the radionuclide to form a complex followed by purification of the complex.

However, although chelating agents such as EDTA and DTPA rapidly chelate the radionuclide, they suffer from the disadvantage that such binding is kinetically labile. In addition, the use of labile radionuclides for antibody labeling in this manner allows substitutionally labile trace metals (which may not be radioactive) to be incorporated into the

chelate. Competition for such non-active trace metals diminishes the biological efficacy of the antibody/chelate complex since a lower quantity of radionuclide is delivered to the target site.

A disadvantage associated with chelating agents such as EDTA and DTPA is the premature release of the chelated radioactive isotope. To lower the rate of metal release *in vivo*, bifunctional chelating agents, based on macrocyclic ligands, such as DOTA (1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid), have been used. See, for example, Mirzadeh et al., ***Bioconjugate Chem.***, 1, 59-65 (1990); and Deshpande et al., ***J. Nucl. Med.***, 31, 473-479 (1990).

A disadvantage in the use of macrocyclic chelating agents is the relatively slow chelation rates which take place at room temperature, see for example, Mirzadeh et al., ***Bioconjugate Chem.***, 1, 59-65 (1990). To reduce the radiolysis which occurs with prolonged chelation times, the medium in which the chelation takes place is often heated to increase the rate of chelation. As the isothiocyanate functionality is heat sensitive, U.S. Patent 5,006,643 discloses the preparation of isocyanato functionalized macrocycles containing primary or secondary amines by first reacting the chelating agent with rhodium and then activating the chelate. U.S. Patent 4,885,363 discloses chelating gadolinium to a tetraazamacrocycle containing tertiary amines prior to activating with an isothiocyanate functional group. Using this procedure for preparation of metal chelate-protein conjugates to be used in radioimmunotherapy or radiodiagnostics requires the formation of the chelate, activation of the chelate and then conjugation of the activated chelate to an antibody in a short period of time to avoid a substantial loss in activity.

Another disadvantage in the use of macrocyclic chelating agents is that during their synthesis and purification, the reagents and materials used must be free of extraneous metal ions. Metal ion contaminants present during these steps may become tightly bound to the macrocycles and not easily displaced during chelation of the desired metal ion. Metal ion contamination during synthesis and purification will reduce the purity of the product obtained during chelation of the desired metal ion as well as reduce the purity of product obtained in subsequent steps in producing the conjugate.

Consequently, it would be advantageous to have a process to prepare purified macrocyclic chelating agents which are substantially pure of contaminating metal ions.

It would also be advantageous to have a process to prepare the isothiocyanate activated chelating agent in an aqueous environment with an increased yield and higher purity than can be prepared when the reaction is performed in an organic solvent/water environment.

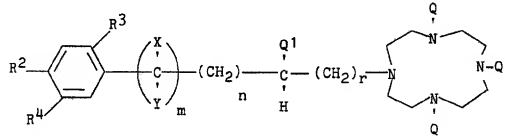
It would be desirable to provide a chelating agent which will react rapidly with a radionuclide at room temperature forming a chelate which does not readily dissociate, allowing the chelating agent to be activated to an isothiocyanate prior to forming the chelate. Activating the chelating agent prior to chelation of the metal ion allows the bifunctional

chelating agent to be prepared in an activated form in advance of when it is needed to produce a conjugate.

It would be further advantageous to have a process which allows rapid conjugation of the chelate with an antibody to reduce radiolysis and antibody aggregate formation. Rapid formation of the conjugate with a reduction in undesired reactions would result in a more complete conjugation reaction and result in easier product purification. Also, having a process wherein the yield and purity from each step is increased is particularly advantageous in preparing compounds and compositions which will be used in pharmaceutical applications where the pharmaceutical composition must meet the purity criteria set-forth by the U. S. Food and Drug Administration.

The present invention provides a chromatographic process for prevention of extraneous metal ion incorporation in a macrocyclic chelating agent during purification. The process is advantageous over existing procedures in that multigram quantities of macrocyclic chelating agent free of divalent cations can be produced and purified in a single unit operation.

The invention also provides a process for preparing isothiocyanate compounds which comprises reacting thiophosgene with a polyaza chelating agent in an aqueous environment in the absence of an organic solvent to form an isothiocyanato derivatized polyaza chelating agent, wherein the polyaza chelating agent is of the formula



(I)

wherein:

each Q is independently hydrogen, $(CHR^5)_pCO_2R$ or $(CHR^5)_pPO_3H_2$;

Q^1 is hydrogen or $(CHR^5)_wCO_2R$;

each R independently is hydrogen, benzyl or C_1-C_4 alkyl;

with the proviso that at least two of the sum of Q and Q^1 must be other than hydrogen;

each R^5 independently is hydrogen, C_1-C_4 alkyl or -(C_1-C_2 alkyl)phenyl;

X and Y are each independently hydrogen or may be taken with an adjacent X and Y to form an additional carbon-carbon bond;

n is 0 or 1;

m is an integer from 0 to 10 inclusive;

p = 1 or 2;

r = 0 or 1;

w = 0 or 1;

with the proviso that n is only 1 when X and/or Y form an additional carbon-carbon bond and the sum of r and w is 0 or 1;

R^2 and R^4 are independently hydrogen, or amino;

R^3 C_1-C_4 alkoxy, $-OCH_2CO_2H$, hydroxy and hydrogen;

with the proviso that R^2 and R^4 cannot both be hydrogen but one of R^2 and R^4 must be hydrogen.

The present invention also describes a process for preparing a conjugate which comprises reacting an isothiocyanato activated chelate with an antibody at between 25°C and 40°C; wherein the chelate is a chelating agent complexed with a metal ion, the chelating agent is as described in Formula I and the metal ion is ^{153}Sm, ^{166}Ho, ^{175}Yb, ^{177}Lu, ^{159}Gd, ^{140}La, ^{142}Pr, ^{149}Pm, ^{90}Y or ^{111}In.

The current invention also provides an improved process for formation of a chelate-antibody conjugate wherein the improvement comprises contacting the chelate with an antibody at 25°C to about 40°C.

The present invention provides an improved process to prepare polyaza metal conjugates. In particular, the present invention is a process for preparing conjugates having at least the steps of:

(a) purifying a polyaza chelating agent, the polyaza chelating agent being as defined in Formula I;

(b) contacting the purified polyaza chelating agent with thiophosgene to form an isothiocyanato activated polyaza chelating agent and recovery of the isothiocyanato activated polyaza chelating agent;

(c) adding a metal ion to the isothiocyanto activated polyaza chelating agent to form an isothiocyanato activated polyaza chelate; and

(d) contacting the isothiocyanato activated polyaza chelate with a biological molecule to form a conjugate;

the improvement which comprises:

in step (a): purifying the polyaza chelating agent by chromatography on a silica gel column, wherein the silica gel has been acid washed prior to loading the polyaza chelating agent onto the column;

in step (b): contacting the purified polyaza chelating agent with thiophosgene in an aqueous environment in the absence of an organic solvent at a pH from 1 to 5; and

in step (d) contacting the isothiocyanato activated polyaza metal chelate with a biological molecule at between 25°C to 40°C.

As used herein, the terms "chelating agent" or "ligand" mean a compound capable of chelating or sequestering a metal ion. The term "chelate" means a chelating agent which has sequestered a metal ion.

The term "bifunctional chelating agent" refers to compounds that have a moiety capable of chelating a metal ion and a linker/spacer moiety covalently bonded to the chelating moiety that is capable of being activated or functionalized to serve as a means to covalently attach to a biological molecule.

The term "biological molecule" refers to any protein, antibody, antibody fragment, hormone, antigen or hapten which functions to recognize a specific biological target site. Such a biological molecule, when attached to a functionalized chelate, serves to carry the attached metal ion to specific targeted tissues. Preferably, the biological material is an antibody or antibody fragment.

As used herein, "antibody" refers to any polyclonal, monoclonal, chimeric antibody, heteroantibody, or recombinant or derivative thereof, preferably a monoclonal antibody. As used herein the term "antibody fragment" includes Fab fragments and $F(ab')_2$ fragments and any portion of an antibody, including recombinants and derivatives thereof, having specificity toward a desired epitope or epitopes. The antibody fragments may be produced by conventional enzymatic methods or by genetic or protein engineering techniques, such as the production of single chain antibodies.

The terms "activated" or "activating" in relation to a chelating agent means the chelating agent has been modified with a functional group which is capable of forming a covalent bond with a biological molecule.

The term "conjugate" as used herein refers to a complex of a biological material attached to a bifunctional chelating agent or bifunctional chelate. The term "antibody/chelate conjugate" refers to an antibody which is covalently attached to a bifunctional chelate, (i.e., the bifunctional chelating agent having a chelated metal ion).

A general overall procedure giving the steps for preparation of conjugates used in the present invention is given in the following Scheme I. Steps (b) and (c) of the process scheme may be reversed. When sequestering metal ions which are radionuclides with short half lives, it is preferred that the activation occurs before formation of a chelate to avoid radiolysis which would occur during the subsequent activating step and any subsequent purification.

Scheme 1

Synthesis of a Chelating Agent

↓

Purification of the Chelating Agent on Silica Gel

Step (a)

↓

Activation of the Chelating Agent

Step (b)

↓

Chelation of a Metal Ion by the Activated Chelating Agent to Form a Chelate

Step (c)

↓

Conjugation of the Chelate to a Biological Material to Form a Conjugate

Step (d)

↓

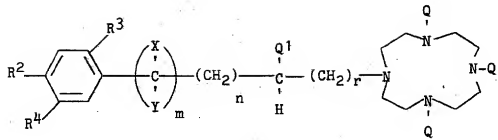
Purification of the Conjugate

The present invention provides a process for reducing extraneous metal ions, specifically calcium ions (Ca^{2+}), during the purification of macrocyclic chelating agents by flash chromatography using silica gel. It has been found that commercially available silica gel used to purify the macrocyclic chelating agents unexpectedly contains a source of undesired metal ions, particularly divalent cations such as calcium, which become tightly bound to the chelating agent during purification. The washing of the silica gel with a strong mineral acid, such as hydrochloric, nitric, sulfuric, perchloric or hydrobromic, prior to use substantially reduces the amount of undesired metal ions bound to the chelating agent during the purification process. Preferably, the mineral acid used to wash the silica gel is hydrochloric acid. Procedures for washing silica gel with a strong acid to remove impurities are known in the art, see, for example, Ralph K. Iler, *The Chemistry of Silica*, John Wiley & Sons (1979).

The elimination of undesired metal ions from the macrocyclic chelating agents during the initial purification results in several advantages in the subsequent steps for forming a conjugate. The elimination of undesired bound metal ions from the macrocyclic chelating agents allows rapid chelation of the desired metal ions at room temperature as opposed to heating or long reaction times required to displace the calcium. The ability to perform the chelation step at room temperature, rather than at temperatures which would destroy the functionalizing group, allows the activation of the macrocyclic bifunctional chelating agent prior to chelation of the metal ion. The ability to functionalize the macrocyclic chelating agent prior to chelation of the metal ion is particularly advantageous as the activated chelating agent can be synthesized, stored and then sequestration of the metal ion performed shortly before conjugation to the biological material. This is particularly advantageous when the metal ion is a radionuclide with a half life of 10 days or less, as a significant amount of radiolysis can occur during the time necessary for functionalizing the chelate, conjugation, and purification of the products from each step. Preclusion of undesired metal ions is particularly important in insuring rapid uptake of radioactive metals of the lanthanide (III) series.

The ability to rapidly chelate the radionuclide after activation of the chelating agent also allows for simpler and more efficient purification procedures to be used in preparation of the conjugate.

The present process for purifying bifunctional chelating agents can be used for many classes of ligands including any tri to hexa -(carboxylated), -(phosphonomethylated), or -(phosphinomethylated) polyazamacrocycle where the polyazamacrocycle has 3 to 6 nitrogens in the ring and the total number of atoms in the ring is 9 to 24 atoms. Of particular interest are aminocarboxylic acid chelating agents, aminophosphonic chelating agents and polyaza chelating agents containing secondary and tertiary amines. Preferred polyazamacrocyclic chelating agents are of the formula



(I)

wherein:

each Q is independently hydrogen, $(CHR^5)_pCO_2R$ or $(CHR^5)_pPO_3H_2$;

Q^1 is hydrogen or $(CHR^5)_wCO_2R$;

each R independently is hydrogen, benzyl or C_1-C_4 alkyl;

with the proviso that that at least two of the sum of Q and Q^1 must be other than hydrogen;

each R^5 independently is hydrogen, C_1-C_4 alkyl or -(C_1-C_2 alkyl)phenyl;

X and Y are each independently hydrogen or may be taken with an adjacent X and Y to form an additional carbon-carbon bond;

n is 0 or 1;

m is an integer from 0 to 10 inclusive;

p = 1 or 2;

r = 0 or 1;

w = 0 or 1;

with the proviso that n is only 1 when X and/or Y form an additional carbon-carbon bond, and the sum of r and w is 0 or 1;

R^2 and R^4 are independently hydrogen, nitro, amino or carboxyl;

R^3 is C_1-C_4 alkoxy, $-OCH_2CO_2H$, hydroxy or hydrogen;

with the proviso that R^2 and R^4 cannot both be hydrogen but one of R^2 and R^4 must be hydrogen.

Preferred amines of the polyaza chelating agents of Formula I are tertiary amines, preferably where r is 0 and each Q is $(CHR^5)_pCO_2R$.

Procedures for synthesizing polyaza macrocycles are well known in the art.

Examples of preferred chelating agents are given in Table I and are named as follows:

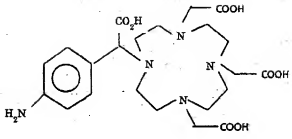
I A is 1-(4-aminophenyl)-1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetraacetic acid;

I B is 1-[2-(4-aminophenyl)ethyl]-1,4,7,10-tetraazacyclododecane--4,7,10-triacetic acid;

I C is 1-[2-(4-aminophenyl)ethyl]-1,4,7,10-tetraazacyclododecane-
-1,4,7,10-tetraacetic acid;
I D is 1-(5-amino-2-methoxybenzyl)-1,4,7,10-tetraazacyclododecane-
-1,4,7,10-tetraacetic acid;
I E is 1-(5-amino-2-hydroxybenzyl)-1,4,7,10-tetraazacyclododecane-
-4,7,10-triacetic acid; and
I F is 1-[2-(4-aminophenyl)ethyl] -1,4,7,10-tetraazacyclododecane-
-1-(R,S,-acetic-4,7,10-tris-(S-methylacetic) acid, the preparation of which is given in European Patent Publication No. 0420942, published April 10, 1991.

The preparation of IA-IE is given in European Patent Publication No. 0353450, published February 7, 1990, and European Patent Publication No. 0420942, published April 10, 1991.

TABLE I



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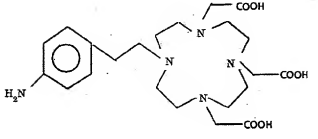

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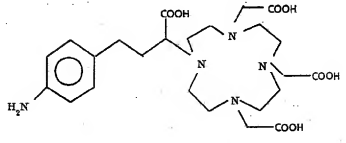

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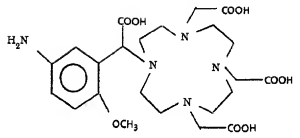
TABLE I CONT'D



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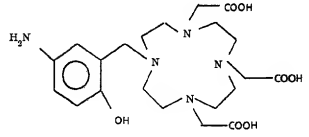

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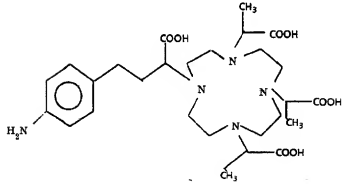


IF

Methods for the carboxylation of an amine of a ligand to give amine derivatives containing a carboxyalkyl group are well known, as are the methods which give alkyl phosphonic and hydroxyalkyl substituents on the amine nitrogens. [See, for example, U.S. Patents 3,726,912 and 3,398,198.]

Aminophosphonic acid derivatives of ligands can be prepared by a number of known synthetic techniques. Of particular importance is the reaction of a compound containing at least one reactive amine hydrogen with a carbonyl compound (aldehyde or ketone) and phosphorous acid or derivative thereof. [See the procedure of Moeoritzer and Irani; *J. Org. Chem.*, 31, 1603 (1966).]

The polyaza chelating agents of the present invention purified by flash chromatography on acid washed silica gel can be activated with any of the known functional groups capable of forming a covalent bond with a biological molecule. Examples of such functional groups are isothiocyanate, bromoacetamide, maleimide, imidoester, thiophthalamide, diazonium and carboxyl. The use of polyaza chelating agents which are essentially free of undesired metal ions are particularly important when using functionalizing groups which are pH and/or thermally unstable, such as isothiocyanate. The activating functional groups are located at positions R^2 or R^4 of Formula I on the macrocyclic chelating agent.

It has been unexpectedly found that when reacting the polyaza chelating agent with thiophosgene to activate the chelating agent with an isothiocyanate group, conducting the reaction in an aqueous environment, preferably in a dilute acid, with vigorous mixing in the absence of an organic solvent results in an activated chelating agent of greater purity than that obtained in the presence of an organic solvent. This is in contrast to the known procedures of using a pH of 8.5 and/or adding the thiophosgene in the presence of an organic solvent. See, for example, Brechbiel et al., *Inorg. Chem.*, 25, 2772-2781 (1986); Keana and Mann, *J. Org. Chem.*, 55, 2868-2871 (1990); Westerberg et al., *J. Med. Chem.*, 32, 236-243 (1989); and Kline et al., *Bioconjugate Chem.*, 2, 26-31 (1991). The reaction of the thiophosgene with the chelating agent is at a pH from 1 to 7. Preferably, the pH is from 1 to 5. More preferably, the pH is from 1 to 3.

The reaction between the chelating agent and thiophosgene is preferably carried out in an aqueous environment having an acid concentration of from about 0.005 to about 0.5 normal (N), preferably 0.01 to 0.2 N. The acid can be any mineral acid, preferably hydrochloric acid. The reaction between the chelating agent and thiophosgene in a dilute acid with vigorous mixing and excess thiophosgene is very fast and usually complete in less than 5 minutes at room temperature (15°C to 25°C). Higher or lower temperatures can be used (e.g., 0°C to 50°C) but room temperature is preferred.

The amount of excess thiophosgene added to the mixture depends on the concentration of the polyaza chelating agent. The lower the concentration of chelating agent, the larger the excess of thiophosgene to insure the rapid and complete conversion of amine to isothiocyanate. For example, if the concentration of chelating agent is 10^{-3} M, the ratio of thiophosgene to chelating agent is 5-20:1. If the concentration of chelating agent is 10^{-8} M, the ratio of thiophosgene to chelating agent is several thousand times larger (i.e., 10^5:1). The excess thiophosgene is removed by conventional techniques such as evaporation, chromatography or extraction.

Rapid mixing of thiophosgene with the aqueous solution may be accomplished using conventional equipment known to those in the art which is capable of producing sufficient shear to produce dispersion of the thiophosgene within the aqueous solution.

Illustrative of such mixing means is the use of a Waring blender for large scale preparations and with a Mixxor™ type mixer available from Alltech Inc., for smaller scale preparations.

By conducting the reaction of the chelating agent with thiophosgene as described above, the obtained purity of the isothiocyanato activated chelating agent is about 90 to about 95 percent as measured by high performance liquid chromatography (HPLC).

After the chelating agent has been activated, the activated chelating agent is placed in contact with a metal ion to form the chelating agent/metal ion chelate. Although any metal ion, whether a radioactive metal ion or not, can be used which is sequestered by the chelating agent, the chelates formed should have reasonable stability such that the metal complex is not readily disassociated. Radionuclides are preferred because of the use of the resulting products in a radiopharmaceutical drug for therapy and/or diagnosis. Especially preferred radioactive isotopes are those of samarium (Sm-153), holmium (Ho-166), ytterbium (Yb-175), lutetium (Lu-177), gadolinium (Gd-159), lanthanum (La-140), praseodymium (Pr-142), promethium (Pm-149). yttrium (Y-90) and indium (In-111).

Radionuclides can be produced in several ways. In a nuclear reactor a nuclide is bombarded with neutrons to obtain a radionuclide, e.g.,

Sm-152 + neutron → Sm-153 + gamma

Another method of obtaining radionuclides is to bombard nuclides with particles produced by a linear accelerator or a cyclotron. Yet another way is to isolate the radionuclide from a mixture of fission products. The method of obtaining the nuclides employed in the present invention is not critical thereto.

The radionuclides can be complexed with the bifunctional chelating agent by adding the bifunctional chelating agent to a solution of the radionuclide. Chelates form readily upon mixing in an aqueous solution at a pH of 1 to 10. Preferably, the reaction is carried out in a medium having a pH of 1 to 7 and more preferably 5 to 7. Ambient temperatures of about 10°C to 40°C can be readily employed for metal ion chelation. The amount of metal ion employed may be from trace amounts to an amount in excess of equimolar with the chelate. Preferably, the formation of the chelate occurs at room temperature, between 15°C to 25°C.

Chelation proceeds rapidly and yields of about 90 percent and greater as measured by high performance liquid chromatography are obtained when using a chelating agent which has been initially purified on substantially calcium free silica gel and activated with thiophosgene prior to chelation of the desired metal ion.

The ability to rapidly form chelates over a wide pH range is advantageous to obtain a high chelation yield of the desired metal ion. When it is necessary to displace divalent cations such as calcium prior to chelation of the desired metal ion, yields are variable due to the pH sensitive nature of the displacement reaction. Displacement of the calcium ion requires running the reaction at a pH sufficiently low to displace the calcium, yet the pH cannot be too low as to adversely affect the chelation of the desired metal ion. Utilizing the procedure of the

present invention allows the chelating reaction to proceed with high yields over a broad pH range.

The ability to activate the chelating agent prior to forming the chelate and the rapid formation of the chelate with a high yield also has the advantage in that it reduces the purification necessary prior to the conjugation step. When the metal ion is chelated prior to activation using thiophosgene, it is necessary to extract the unreacted thiophosgene with an organic solvent and then purify the chelate prior to conjugation. The low concentration of the chelate in the elution volume obtained after purification using an ion exchange column necessitates concentrating the chelate prior to conjugation. During the process of extracting the thiophosgene and concentrating the eluent by reduced pressure and/or heat, some degradation of the product occurs along with radiolysis creating additional impurities. Following the chelation step of the present invention, the activated chelate can be rapidly purified by column chromatography and the eluent used directly, without concentration, in the conjugation step.

The bifunctional chelate is preferably conjugated to a biological molecule which carries out a specific target function. In a preferred embodiment, the biomolecule is a monoclonal antibody or fragment thereof which is specific against a selected cell-surface target site. Such antibodies may be commercially available or may be made by standard somatic cell hybridization techniques. An example of a suitable monoclonal antibody is CC49, one of a series of monoclonal antibodies specific for TAG-72 (tumor associated glycoprotein) described in published PCT Application No. WO 89/00692, on January 26, 1989, and published PCT application WO 90/04410, on May 3, 1990. Generally, the chelate and protein are mixed in a molar ratio of greater than 1:10 and less than about 100:1 depending on the protein and protein concentration. Ratios of 0.5:1 to 4:1 are preferred.

Methods for conjugating thiocyanate derivatized chelates to antibodies are well known in the art. The procedure generally involves reacting the functionalized chelating agent or chelate with the antibody from 2 to 18 hours in an aqueous buffered solution at pH 6-10 at room temperature.

The increase in purity of the chelate obtained by the process of the present invention allows the chelate to be conjugated to a biological material, preferably a protein, at temperatures from 25°C to 40°C, preferably 30°C to 40°C, to obtain a protein/chelate containing an activity from 0.5 millicuries to 30 millicuries per mg protein without the expected increase in degradation of the chelate activated group (i.e., hydrolysis of the isothiocyanate) or protein. As the temperature of the chelation reaction is increased, an increase in the rate of formation of protein aggregates and hydrolysis of the chelate activated groups would be expected in addition to an increased conjugation rate. Unexpectedly, elevated temperature results in a much faster conjugation rate increase relative to the rate of increase of undesired by-product formation (e.g., chelate hydrolysis and protein aggregates). Performing the conjugation at

about pH 9.5 and 37°C, the reaction is approximately 92 percent complete in one hour with less than 4 percent of the radioactivity being associated with protein aggregates.

The formation of fewer by-products during the conjugation coupled with a more complete reaction leads to easier product purification, production of a product with higher purity, and gives a greater overall recovery of the initial radioactivity. Utilizing a conjugation temperature of 37°C also allows a radiochemical yield of greater than 90 percent during purification by column chromatography as compared to a radiochemical yield of approximately 80 percent when the conjugation is done at 20°C.

The invention will be further clarified by a consideration of the following examples, which are intended to be purely exemplary of the present invention.

In the following examples, the following terms are used.

BFC = bifunctional chelating agent.

PA-DOTA = 1-[2-(4-aminophenyl)ethyl]-1,4,7,10-
-tetraazacyclododecane-1,4,7,10-tetraacetic acid.

HEPES = N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid.

General Experimental

Mass spectra (fast atom bombardment with xenon) were obtained on a Vacuum Generators ZAB HS mass spectrometer. Samples for mass spectral analysis were prepared by dissolution in a 3:1 mixture of dithiothreitol:dithioerythritol (magic bullet) unless otherwise stated.

Analytical high performance liquid chromatograph (HPLC) of non-radioactive samples was performed on a Hewlett Packard 1090 liquid chromatograph with a 4.6 x 100 mm Alltech Econosphere C18 3 μ column with a flow rate of 1 mL per minute and detection at 254 nm. Samples were eluted with a gradient of 0.05 M, pH 6.0, sodium acetate/ acetonitrile as given for the samples.

HPLC of radioactive samples (i.e., those in which ^{177}Lu was incorporated) was performed using a DuPont Zorbax GF-250 (9.4 x 250 mm) column with a flow rate of 1.5 mL per minute and radioactivity detection. The mobile phase was 0.25 M, pH 6.0 sodium citrate/10 percent acetonitrile.

Example 1 Purification of PA-DOTA by Flash Chromatography

A 1.5 x 14 inch flash chromatography column was packed with 17.0 g of Merck 60 A silica gel, 230-400 mesh (Aldrich Chemical Co.) which had been acid-washed by Alltech Inc. Crude PA-H_4DOTA•3HCl (3.2 g, 75 percent pure by HPLC analysis) was applied to the column and eluted with a mobile phase of 2:2:1 chloroform:methanol:concentrated ammonium hydroxide. Fractions containing only PA-DOTA$(NH_4)_2$ as assayed by thin layer chromatography were collected and pooled to give PA-DOTA$(NH_4)_2$ which was greater than 98 percent area purity as assayed by HPLC analysis. The absence of calcium in the recovered

PA-DOTA(NH_4)$_2$ was confirmed by mass spectrometry and HPLC using an elution buffer gradient of 95/5 sodium acetate (0.05 M, pH = 6)/acetonitrile to 30/70 in 15 minutes.

Chelation of yttrium to the PA-DOTA obtained above was performed by dissolving 3 mg of PA-DOTA in 3 mL of 0.5 of sodium acetate buffer (pH = 6) and 0.2 mL of 40 millimolar $Y(OAC)_3 \cdot 4H_2O$, this represents approximately a 5 fold excess of yttrium. The extent of chelation was monitored by HPLC as described above.

Chelation with PA-DOTA which was purified using acid washed silica gel was complete in less than 5 minutes at room temperature (18-25°C).

Comparative Example A

Chelation with PA-DOTA purified using silica gel which had not been acid washed was 4 percent complete in 15 minutes at room temperature and was 86 percent complete in 10 minutes at 90°C.

These results show acid washing the silica gel to remove extraneous metal ions prior to use for purifying the chelating agent greatly enhances the rate at which the desired metal is sequestered at room temperature.

Example 2 Preparation of SCN-PA-H_4DOTA•2HCl•2NH_4Cl

To 100 mL of 0.01 M hydrochloric acid was added 250 mg of calcium free PA-H_2(NH_4)$_2$DOTA (0.448 millimoles, 2 of the carboxylated groups are protonated and 2 of the carboxylated groups are ammonium salts) as prepared in Example 1, and the solution placed in a 40-oz. Waring blender. Thiophosgene (170 microliters, 2.23 millimoles) was added and the blender quickly started. After 2 minutes of mixing, the mixture was added to a separatory funnel and excess thiophosgene was extracted with three 50 mL portions of chloroform. The aqueous layer was added to 100 mL of acetonitrile and the solution reduced to dryness on a rotary evaporator (fitted with a vacuum pump) at room temperature. The solid obtained was further dried for 2 hours at room temperature on a vacuum line. The yield was 321 mg (90 percent yield) of an off-white solid.

Characterization of the product by mass spectrometry and by HPLC showed the product was 96 percent by weight an isothiocyanate derivatized PA-DOTA (SCN-PA-H_4DOTA•2HCl•2NH_4Cl). The HPLC elution gradient being sodium acetate/acetonitrile 95/5 to 70/30 in 15 minutes and then to 40/60 in 20 minutes.

Comparative Example B

Samples prepared in an identical manner except that the reaction was carried out by the addition of 20 to 50 percent by volume chloroform prior to mixing were 84 to 89 percent pure as measured by HPLC.

These results show that a high purity isothiocyanato activated chelating agent can be obtained by mixing the thiophosgene and chelating agent in an aqueous system in the absence of an organic solvent.

Example 3 Preparation of [^{177}Lu(SCN-A-DOTA)]$^{+}$

To 20 µL of SCN-PA-DOTA (4.88 x 10^{-3} moles) prepared as described in Example 2 was added 100 µL of Lu-177 (50 microcurries) to give a molar ratio of chelating agent to Lu-177 of about 1:1. The solution was mixed on a vortex mixer for about 5 seconds, 100 µL of HEPES buffer (0.5 M, pH 7) were added and the solution mixed for 5 minutes. The pH was measured and adjusted to between pH 6-7 with HEPES buffer if necessary. The reaction was allowed to proceed for another 5 minutes at room temperature and the yield of [^{177}Lu(SCN-A-DOTA)]$^{+}$ was 91.4 percent as determined by HPLC.

The [^{177}Lu(SCN-PA-DOTA)]$^{+}$ complex was purified by placing the sample on a PRP-1 mini-clean™ column (80 µL) which had been pretreated with 800 µL acetonitrile, 400 µL of water and 800 µL 10 percent by volume acetonitrile in 20 mM carbonate buffer, pH 9.5. After loading the complex onto the column, the reaction vial was rinsed with a 200 µL and then a 600 µL volume of 10 percent acetonitrile in carbonate buffer, the washes also being placed on the column.

The complex was then eluted from the column using a 1:2 ratio of carbonate buffer (20 mM, pH 9.5):acetonitrile. Approximately 80 percent of the radioactivity was recovered in the second 50 µL elution volume.

Example 4 Conjugation of [^{177}Lu(SCN-PA-DOTA)]$^{+}$ With IgG CC49 at Room Temperature (21-22°C)

To 257 µL of an antibody solution (IgG CC49, 14.5 mg/mL in 50 mM carbonate buffer, pH 9.5) was added 25 µL of [^{177}Lu(SCN-PA-DOTA)]$^{+}$ prepared as described above, giving a BFC:antibody molar ratio of about 1.36. The activated chelate and antibody solution was mixed on a vortex mixer for about 10 seconds and allowed to stand at room temperature for approximately 2 hours with mixing about every 10 to 15 minutes. The disappearance of the [^{177}Lu(SCN-PA-DOTA)]$^{+}$ was measured by HPLC. After 120 minutes, about 62 percent of the ^{177}Lu activity was associated with the antibody, about 7 percent was non-antibody associated impurities, and unreacted [^{177}Lu(SCN-PA-DOTA)]$^{+}$ was about 30 percent.

Example 5 Conjugation of [^{177}Lu(SCN-PA-DOTA)]$^{+}$ With IGg CC49 at 37°C

To an antibody solution (15.7 mg CC49 IgG (30 nanomoles) in 286 µL of carbonate buffer, 20 mM, pH 9.5) was added 21 nanomoles of [^{177}Lu(SCN-PA-DOTA)]$^{+}$ complex (9.7 millicuries of activity) in 11 µL of the eluent from Example 3. The mixture was mixed on a vortex mixer and allowed to stand in a 37°C oven for one hour with mixing about every 10 to 15 minutes. The progress of the reaction was monitored by HPLC analysis for the disappearance of the complex as described in Example 4. After 1 hour the ^{177}Lu activity associated with the antibody as measured by HPLC was 91 percent, less than 4 percent of the ^{177}Lu activity was associated with impurities, and 5 percent was unreacted [^{177}Lu(SCN-PA-DOTA)]$^{+}$.

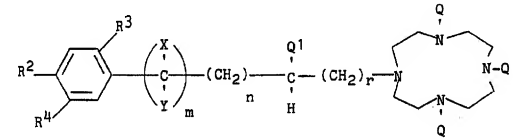
The conjugation reaction was terminated by isolation and purification of the conjugate on a PD-10 column (Sephadex G-25, medium, 9 mL) which had been equilibrated with phosphate buffered saline.

Homogeneity of the labeled antibody was examined by HPLC and by SDS PAGE electrophoresis (sodium dodecyl sulfate-polyacrylamide electrophoresis) coupled with autoradiography. Immunoreactivity was determined by IRMA and affinity column binding and was found to be comparable to that conjugated at 25°C. *In vivo* biodistribution (in rats) of the conjugate prepared at 37°C was not significantly different from that prepared at 25°C.

The results show that the chelate can be rapidly conjugated to an antibody at 37°C without affecting the immunoreactivity or biodistribution of the conjugate.

Other embodiments of the invention will be apparent to those skilled in the art from a consideration of this specification or practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with the true scope and spirit of the invention being indicated by the following claims.

1. A process for preparing a chelate-antibody conjugate which comprises reacting an isothiocyante activated chelate with an antibody at between 30°C and 40°C wherein the chelate is a chelating agent complexed with a metal ion; the chelating agent is of the formula



(I)

wherein:

each Q is independently hydrogen, $(CHR^5)_pCO_2R$ or $(CHR^5)_pPO_3H_2$;

Q^1 is hydrogen or $(CHR^5)_wCO_2R$;

each R independently is hydrogen, benzyl or C_1-C_4 alkyl;

with the proviso that at least two of the sum of Q and Q^1 must be other than hydrogen;

each R^5 independently is hydrogen, C_1-C_4 alkyl or -(C_1-C_2 alkyl)phenyl;

X and Y are each independently hydrogen or may be taken with an adjacent X and Y to form an additional carbon-carbon bond;

n is 0 or 1;

m is an integer from 0 to 10 inclusive;

p = 1 or 2;

r = 0 or 1;

w = 0 or 1;

with the proviso that n is only 1 when X and/or Y form an additional carbon-carbon bond and the sum of r and w is 0 or 1;

R^2 and R^4 are independently hydrogen, or amino;

R^3 C_1-C_4 alkoxy, $-OCH_2CO_2H$, hydroxy and hydrogen;

with the proviso that R^2 and R^4 cannot both be hydrogen but one of R^2 and R^4 must be hydrogen; and the metal ion is selected from the group consisting of ^{153}Sm, ^{166}Ho, ^{175}Yb, ^{177}Lu, ^{159}Gd, ^{140}La, ^{142}Pr, ^{149}Pm, ^{90}Y and ^{111}In.

2. The process of Claim 1 wherein the metal ion is ^{177}Lu.

3. The process of Claim 1 or 2 wherein the chelating agent is 1-[2-(4-aminophenyl)ethyl]-1,4,7,10--tetraazacyclododecane-1,4,7,10-tetraacetic acid.

4. In a process for preparing polyaza conjugates having at least the steps of

(a) purifying a polyaza chelating agent, the polyaza chelating agent being as defined in Formula I;

(b) contacting the the purified polyaza chelating agent with thiophosgene to form an isothiocyanato activated polyaza chelating agent and recovery of the isothiocyanato activated polyaza chelating agent;

(c) adding a metal ion to the isothiocyanto activated polyaza chelating agent to form an isothiocyanato activated polyaza chelate; and

(d) contacting the isothiocyanato activated polyaza chelate with a biological molecule to form a conjugate;

the improvement which comprises;

in step (a): purifying the polyaza chelating agent by chromatography on a silica gel column, wherein the silica gel has been acid washed prior to loading the polyaza chelating agent onto the column;

in step (b): contacting the purified polyaza chelating agent with thiophosgene in an aqueous environment in the absence of an organic solvent at a pH from 1 to 5; and

in step (d): contacting the isothiocyanato activated polyaza chelate with a biological molecule at between 25°C to 40°C.

5. The process of Claim 4 wherein the biological molecule is a protein and the metal ion is ^{153}Sm, ^{166}Ho, ^{175}Yb, ^{177}Lu, ^{159}Gd, ^{140}La, ^{142}Pr, ^{149}Pm, ^{90}Y or ^{111}In.

6. The process of Claim 5 wherein the protein is an antibody.

7. The process of Claim 4, 5 or 6 wherein r of Formula I is 0 and each Q is $(CHR^5)_pCO_2R$.

8. The process of Claim 7 wherein the chelating agent is α-[2-(4-amino-phenyl)ethyl]-1,4,7,10--tetraazacyclododecane-1,4,7,10-tetraacetic acid and the metal ion is ^{177}Lu.

9. In a process for preparing polyaza conjugates having at least the steps of

(a) purifying a polyaza chelating agent, the polyaza chelating agent being as defined in Formula I;

(b) adding a metal ion to the polyaza chelating agent to form a polyaza chelate;

(c) contacting the chelate with thiophosgene to form an isothiocyanato activated chelate

(d) contacting the isothiocyanato activated chelate with a biological molecule to form a conjugate;

the improvement which comprises;

in step (a): purifying the polyaza chelating agent by chromatography on a silica gel column, wherein the silica gel has been acid washed prior to loading the polyaza chelating agent onto the column;

in step (c): contacting the chelate with thiophosgene in an aqueous environment in the absence of an organic solvent at a pH from 1 to 5; and

in step (d): contacting the isothiocyanato activated polyaza chelate with a biological molecule at between 25°C to 40°C.

10. A process for purifying a tri to hexa-(carboxylated), -(phosphonomethylated), or -(phosphinomethylated) polyazamacrocyclic chelating agent comprising flash chromatography of a polyazamacrocycle on acid washed silica gel to give a polyazamacrocycle substantially free of metal ions; wherein the polyazamacrocycle has 3 to 6 nitrogens in the ring and the total number of atoms in the ring is 9 to 24 atoms.